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Peripheral Blood Regulatory T-cell and Type-1 Helper T-cell Population Decrease after Hepatic Artery Embolization

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Abstract

Purpose—To evaluate changes in T-cell populations in peripheral blood following bland hepatic artery embolization HAE).

Materials and Methods—Twelve patients received bland HAE to treat primary ($n=5$) or metastatic ($n=7$) liver tumors, using microsphere and polyvinyl alcohol ($n=8$) or microsphere alone ($n=4$). Patient peripheral blood samples were collected within 1 month before HAE, within 1 week after HAE (early post-HAE period), and 2–8 weeks after HAE (follow-up period). Peripheral blood populations of cytotoxic T-cells (CTL), CD4⁺ T-cells: type-1 (T_h1) and type-2 helper T-cells (T_h2), and regulatory T-cells (T_{reg}) were evaluated using flow cytometry. Changes in T-cell populations before and after bland HAE were compared using paired *t*-tests.

Results—Peripheral blood CD4⁺ T-cell populations decreased significantly both in the early post-HAE (44.0±2.2 to 34.4±3.6%, $p < .01$) and on follow-up (44.0±2.2 to 36.3±3.0%, $p < .01$). Among the individual CD4⁺ T-cell subtypes, T_{reg} (2.5±0.3 to 1.7±0.2%, $p < .02$) and T_h1 (8.1±1.8 to 5.6±1.6%, $p < .02$) populations decreased significantly in the early post-HAE period only. The presence of extrahepatic disease was associated with decreasing T_{reg} population ($p < .04$).

Conclusion—Following HAE, the peripheral blood T-cell environment is changed with decrease in both T_{reg} and T_h1 populations.

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INTRODUCTION

Traditionally, hepatic artery embolization (HAE) efficacy has been attributed mainly to its direct ischemic tumoricidal effect (1,2). Little attention has been devoted to the potential for immune environment. Actually, HAE induces tumor necrosis *in situ* by blocking the tumor blood supply. Therefore, it can create a source of tumor-associated antigen (3,4). Indeed, previous reports of the literature have shown that tumor-specific T-cell response increases after transcatheter arterial chemoembolization (3,4). Moreover, HAE induces local inflammation within the liver leading to the release of various cytokines and chemokines (5–8). Induction of tumor-associated antigens as well as the release of cytokines and chemokines might change the systemic T-cell environment. Therefore, we hypothesize that HAE can alter the peripheral blood T-cell environment. Elucidating the changes in T-cell environment is important because cumulative evidence suggests that T-cell mediated immune response affects the disease progression and patient prognosis for cancer of several types (9–15). However, the effects of HAE on the T-cell environment have not been elucidated satisfactorily in earlier reports of the literature.

For this study, we evaluated changes in peripheral blood T-cell subtypes following bland HAE.

MATERIALS AND METHODS

Patients

This single-center prospective study was approved by our institutional review board. Written informed consent to participation was obtained from all participants who underwent HAE for the treatment of primary or secondary malignancy. Exclusion criteria included prior ablation or embolization procedures within 30 days and patients who did not require a blood draw before the procedure at the time they were evaluated for eligibility.

Between January 2014 and November 2014, five interventional radiologists (AC, GG, KB, SB, and JE) and one research assistant (CT) recruited study participant, and 20 patients were enrolled. In 7 of these 20 patients, scheduled laboratory testing was not performed after the procedure. In addition, the blood sample was insufficient for analysis in 1 patient, leaving 12 patients who formed the basis of this study.

Subjects were 9 men (75.0%) and 3 women (25.0%) with mean age of 56.2 ± 3.6 (range 39–75) years. The most common primary diagnosis was neuroendocrine tumor (50.0%, 6/12), followed by hepatocellular carcinoma (HCC) (41.7%, 5/12) and leiomyosarcoma (8.3%, 1/12). Extrahepatic metastases were present in 6 patients (50.0%, 6/12). Patient and tumor characteristics are presented in Table 1. No patient received other cancer therapy for 30 days or more before study enrollment until the end of follow-up.

Hepatic artery embolization

Bland HAE was performed by five interventional radiologists (AC, GG, KB, SB, and JE). After routine hepatic angiography with a 4-french or 5-french angiographic catheters to identify hepatic arterial anatomy, tumor location, and feeding arteries, bland HAE was

performed via the common femoral artery. Feeding arteries that supplied the target lesion(s) were catheterized as selectively as possible using microcatheters. Bland HAE was conducted using 40–100 μm microspheres (Embosphere®; Biosphere Medical Inc., Rockland, MA) in five patients, 100–300 μm microspheres in four patients, and both 40–100 μm and 100–300 μm microspheres in three patients. The endpoint of embolization was complete stasis of antegrade blood flow in the feeding arteries. To achieve complete stasis, additional embolization by polyvinyl alcohol (PVA) (PVA embolization particles; Cook, Inc., Bloomington, IN) was done in 4 of 5 patients who received HAE by 40–100 μm microspheres, 1 of 4 patients by 100–300 μm , and all three patients who received HAE by both 40–100 μm and 100–300 μm microspheres. If multiple lesions were present in the liver and selection of multiple feeding arteries was difficult, then lobar HAE was performed. Four patients received segmental HAEs by 40–100 μm microspheres plus PVA ($n=3$) or 40–100 μm microspheres alone ($n=1$). The remaining 8 patients received lobar HAE.

Blood collection

Patient peripheral blood was collected within 1 month before HAE, during the early post-HAE period (i.e. within 1 week after HAE), and during the follow-up period (i.e., 2–8 weeks after HAE). This timing of blood collection was chosen according to the previous study, which evaluated the serial changes in serum cytokines after HAE (6). In all, 20 mL peripheral blood was collected at each time point. Assessments of white blood cells (WBC) were performed using fresh blood samples at our clinical chemistry laboratories. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using density gradient centrifugation medium (Ficoll-Paque; GE Healthcare Bio-Sciences, NJ, USA). Then, PBMCs were frozen immediately with freezing medium (Cell Freezing Medium-DMSO 1 \times ; Sigma-Aldrich Corp., MO, USA) at $-80\text{ }^{\circ}\text{C}$ and were stored until analysis.

Surface and intracellular staining

Surface and intracellular staining were conducted to analyze T-cell subtypes by three researchers (HT, NI, and SG). After thawing of frozen PBMCs, 2×10^6 cells were prepared for each analysis. Among the 2×10^6 PBMCs, 1×10^6 cells were used to analyze cytotoxic T-lymphocyte (CTL), CD4^+ T-cells, and regulatory T cells (T_{reg}). PBMCs were first incubated for surface staining with the following antibodies: CD4 PerCP-Cy5.5 (clone SK3; BD Biosciences, CA, USA), CD8 FITC (clone HIT8a; BD Pharmingen, CA, USA), and CD25 (clone 4E3; Miltenyi Biotec, CA, USA). After washing the cells using phosphate buffered saline with 2% fetal bovine serum, intracellular staining by forkhead/winged helix transcription factor (FoxP3) PE (clone 236A/E7; eBioscience, CA, USA) was done according to instructions provided by the manufacturer (Anti-Human FOXP3 Staining Set PE; eBioscience, CA, USA).

Remaining 1×10^6 PBMCs were used for the analysis of Type-1 ($\text{T}_{\text{H}}1$) and Type-2 helper T-cells ($\text{T}_{\text{H}}2$). After surface staining with antibodies for CD4 PerCP-Cy5.5, cells were stimulated with 10 ng/ml of phorbol myristate acetate (PMA; Sigma-Aldrich Co. LLC) and 1 ng/ml of ionomycin (Sigma-Aldrich Co. LLC) in the presence of 0.67 $\mu\text{l/ml}$ of protein transport inhibitor containing monensin (BD Golgistop; BD Biosciences) for 4 hr at $37\text{ }^{\circ}\text{C}$ in an atmosphere containing 5% CO_2 . Then, intracellular staining was done for interferon

gamma (IFN- γ) FITC (Clone B27; BD Pharmingen) and interleukin 4 (IL-4) APC (Clone MP4-25D2; BD Pharmingen).

Flow cytometry

Populations of each T-cell subset were analyzed using flow cytometry (FACSCalibur; Becton, Dickinson and Co.) (Figs. 1,2). After the lymphocyte population was gated from PBMCs according to forward scatter and side scatter characteristics, 20,000 lymphocytes were collected in each sample. Then, CD4⁺ and CD8⁺ subsets were gated by referencing the unstained sample. Populations of CD25⁺FoxP3⁺, IFN- γ ⁺, and IL-4⁺ cells were evaluated further using CD4⁺ gate. Isotype-matched PE-, PerCP-Cy5.5-, APC-, and FITC-conjugated mouse IgG₁ antibodies were used as controls (BD Biosciences, BD Pharmingen, and Miltenyi Biotec). All flow cytometry analyses were performed using software (BD CellQuest; Becton, Dickinson and Co.).

Measurements and statistical analysis

This study evaluated the serial change of peripheral blood WBCs, neutrophils, and lymphocytes after HAE. We also evaluated the percentages of T-cell subtypes including CD8⁺ CTL, CD4⁺ T-cells, CD4⁺CD25⁺Foxp3⁺ T_{reg}, CD4⁺IFN- γ ⁺ T_h1, and CD4⁺IL-4⁺ T_h2.

Data are expressed as mean and standard error (SE). Comparisons of samples between time points were made using paired *t* tests. If significant changes in T-cell population were found after HAE, factors affecting these changes were also evaluated. Because of variation of the baseline T-cell population, we used fold-change for this evaluation, defined as the post/pre ratio of T-cell population. Comparisons of fold-changes of T-cell subtypes between subgroups (i.e., patient backgrounds, tumor characteristics, characteristics at the time of HAE) were done using Mann–Whitney U tests. All *p* values less than .05 were inferred as significant. All statistical analyses were conducted using software (SAS, release 9.1; SAS Institute Inc., Cary, NC, USA).

RESULTS

Changes in white blood cell, neutrophil, and lymphocyte counts

The intervals between HAE and post-HAE blood samplings were 1.2±0.6 days (early post-HAE period; range 1–3 days) and 34.8±14.2 (follow-up period; range 14–62 days), respectively. Changes in WBC, neutrophil, and lymphocyte counts are presented in Figs. 3a–3e. WBC counts (6.5 ± 1.1 to 9.6 ± 1.4 ×10³/μL, *p* < .01), neutrophil populations (63.9 ± 3.5 to 77.2 ± 3.7%, *p* < .01), and neutrophil counts (3.8 ± 0.4 to 7.5 ± 1.1 ×10³/μL, *p* < .01) increased significantly during the early post-HAE period (Figs. 3a–3c). Lymphocyte populations (25.7 ± 3.5 to 14.7 ± 3.5%, *p* < .01) and counts (2.0 ± 0.7 to 1.4±0.6 ×10³/μL, *p* < .02) decreased significantly during the early post-HAE period (Figs. 3d, 3e). All of those changes returned to baseline levels during the follow-up period (Figs. 3a–3e).

Changes in T-cell population

Changes in the populations of T-cell subtypes are presented in Figs. 4a–4e. A significant decrease of CD4⁺ T-cell population was found in both the early post-HAE period (44.0±2.2

to $34.4 \pm 3.6\%$, $p < .01$) and the follow-up periods (44.0 ± 2.2 to $36.3 \pm 3.0\%$, $p < .01$) (Fig. 4b). Among the individual CD4⁺ T-cell subtypes, T_{reg} (2.5 ± 0.3 to $1.7 \pm 0.2\%$, $p < .02$) and T_{h1} (8.1 ± 1.8 to $5.6 \pm 1.6\%$, $p < .02$) populations decreased significantly in the early post-HAE period (Figs. 4c, 4e). However, no significant change was observed in any population of CTL and T_{h2} (Figs. 4a, 4e).

Factors affecting fold-changes of T_{reg} and T_{h1} were also evaluated (Table 2). The presence of extrahepatic disease was associated with decreasing T_{reg} population in early post-HAE periods ($p < .04$).

DISCUSSION

Results of our study have demonstrated that peripheral blood lymphocytes decrease and that neutrophils increase in early post-HAE periods. This change, which is commonly observed after HAE, reflects systemic inflammatory change (16). It is more interesting that results of our study verified the hypothesis that HAE can alter the peripheral blood T-cell environment.

In this study, the peripheral blood T_{reg} population decreased after HAE. T_{reg} is a T-cell subtype that maintains self-tolerance and homeostasis in the immune system (17). Results of several studies show that the tumor microenvironment can promote Treg induction and recruitment (18), and show that the frequency of peripheral T_{reg} increases with tumor progression (19,20). The T_{reg} population is known to correlate with the tumor burden (19,20). Therefore, the decrease of T_{reg} population after HAE observed in this study might simply reflect the decrease of the tumor burden. Another explanation of the T_{reg} decrease might be the altered cytokine environment. Previous studies have reported that a robust increase of IL-6 was observed in the early post-HAE period (6–8). Reportedly, IL-6 inhibits transforming growth-factor- β -induced T_{reg} differentiation (21).

In cancer patients, T_{reg} is known to suppress anti-tumor immune response. Increased T_{reg} population is associated with poorer prognosis (22–25). Therefore, a decrease in the T_{reg} population might benefit the enhancement of systemic anti-tumor immunity. Indeed, various laboratory and clinical reports have described that T_{reg} depletion increase systemic antitumor effects (26,27).

T_{h1} is another subtype of T-cell which enhances antitumor immune response by secreting IFN- γ and activating CTL. Therefore, a decrease of T_{h1} can negatively affect antitumor immunity. In this study, peripheral blood T_{h1} population also decreased after HAE. The decrease of T_{h1} population might also be related to the cytokine environment. Differentiation of T_{h1} from naïve T-cells is inhibited by IL-6 (28). Therefore, increased IL-6 after HAE might engender the decrease of the peripheral blood T_{h1} population, although further studies must be conducted to confirm this hypothesis.

It is noteworthy that altered populations of lymphocyte and T-cell subtypes in the early post-HAE period returned to the baseline levels in the follow-up period. This phenomenon might also be related to the cytokine environment. Previous reports have described that elevated IL-6 levels returned to the baseline at 1 month after embolization (6).

Given that the decrease of T_{reg} population was transient and that the T_{h1} population also decreased in this study, HAE might affect the systemic antitumor immune response both positively and negatively. Indeed, spontaneous regression of extra-hepatic metastasis is rarely observed after HAE (29). However, results of this study suggest an adjuvant approach to enhance antitumor effects. Systemic antitumor immune response after HAE might be reinforced if adjuvant therapies which sustain T_{reg} decrease and simultaneously boost T_{h1} response could be combined. Previous reports have described that T_{h1} response enhancement and T_{reg} depletion increase antitumor effects (26,27,30).

This study has several limitations that include the small number of enrolled patients, the high drop-out rate of enrolled patients, the heterogeneous patient backgrounds and HAE technique, the lack of a control group, and short follow-up periods. Moreover, the time points of blood sampling were limited and timing was wide ranged especially during the follow-up period. This variation might confound study results. However, the primary purpose of this study was to report preliminary data of changes in T-cell subtypes after HAE. Additional research with a stricter schedule of blood sampling must be undertaken to confirm the results of this study. Third, we only evaluated peripheral blood T-cells; those in the tumor microenvironment were not evaluated. Fourth, although minor comorbidities such as the common cold might also affect peripheral blood T-cell populations, effects of such comorbidities were not examined closely. Fifth, we did not evaluate the antigen-specific T-cell response. Therefore, it remains unclear if changes in T-cell populations observed in this study reflect antigen-specific T-cell response. Finally, we only evaluated changes in the peripheral blood T-cell subtypes after HAE. Relations with tumor progression and patient prognosis were not evaluated. Therefore, the clinical relevance of T-cell changes observed in this study remains unclear. Despite these limitations, results of our study yielded useful preliminary data for changes in T-cell subtypes after HAE.

In conclusion, HAE can alter the peripheral blood T-cell environment by decreasing both T_{reg} and T_{h1} populations.

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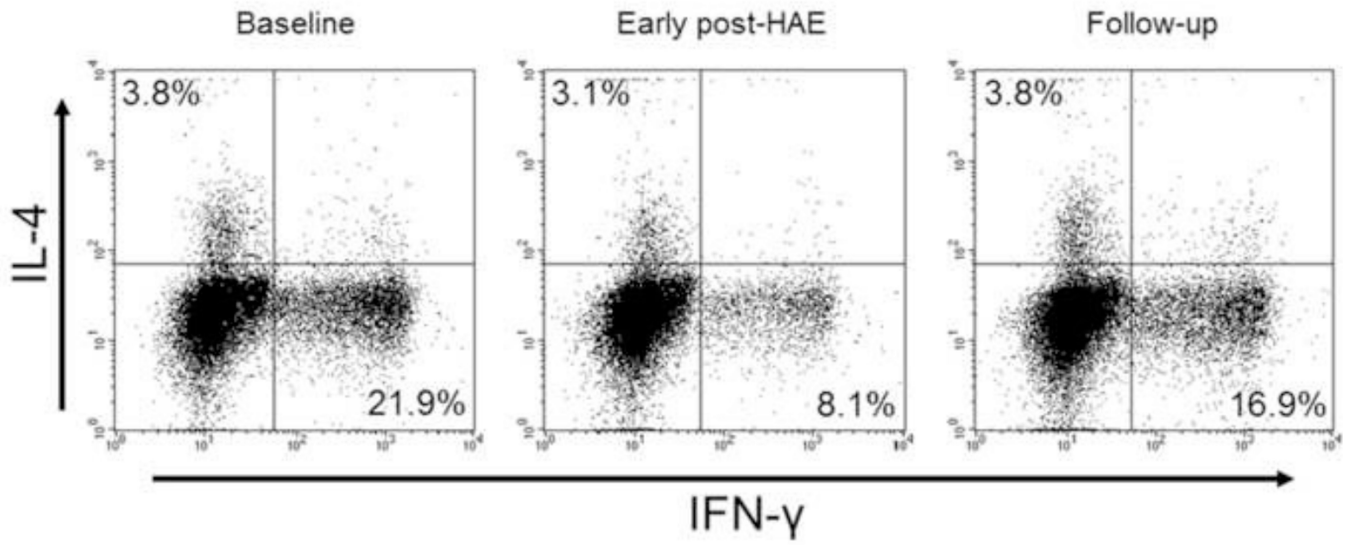


Figure 1.

Representative flow cytometry results for a patient who received hepatic artery embolization (HAE) for the treatment of hepatocellular carcinoma. Population of peripheral blood Type-1 helper T-cell (T_H1) was decreased (21.9% to 8.1%) in the early post-HAE period. It returned to the baseline level (16.9%) during the follow-up period.

IL-4, interleukin-4; IFN- γ , interferon-gamma

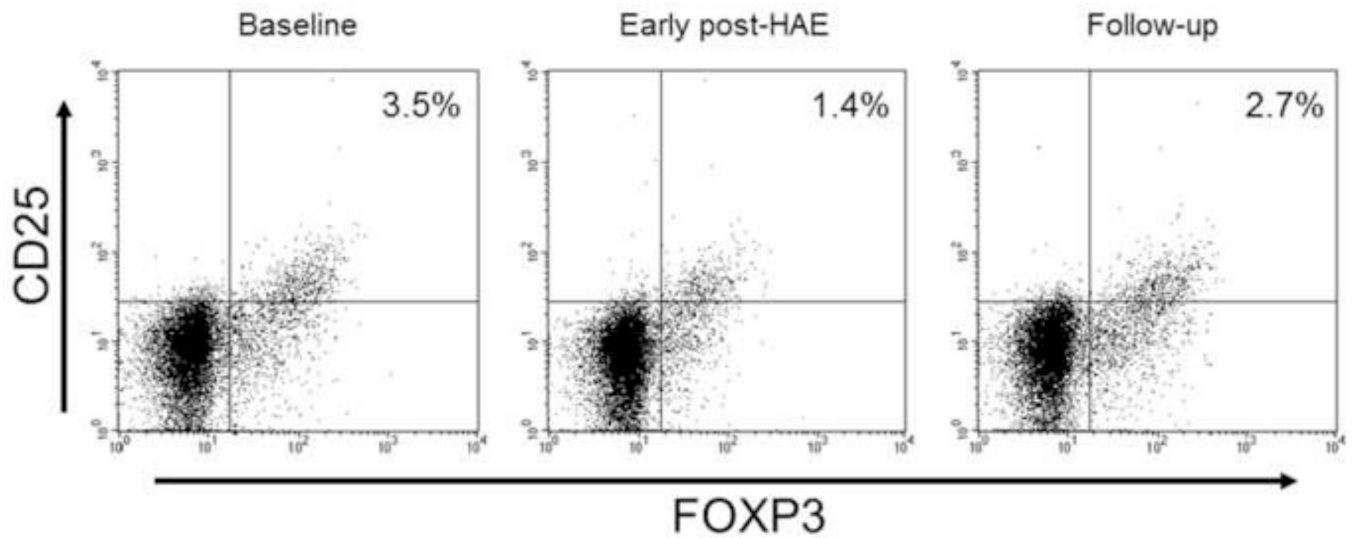
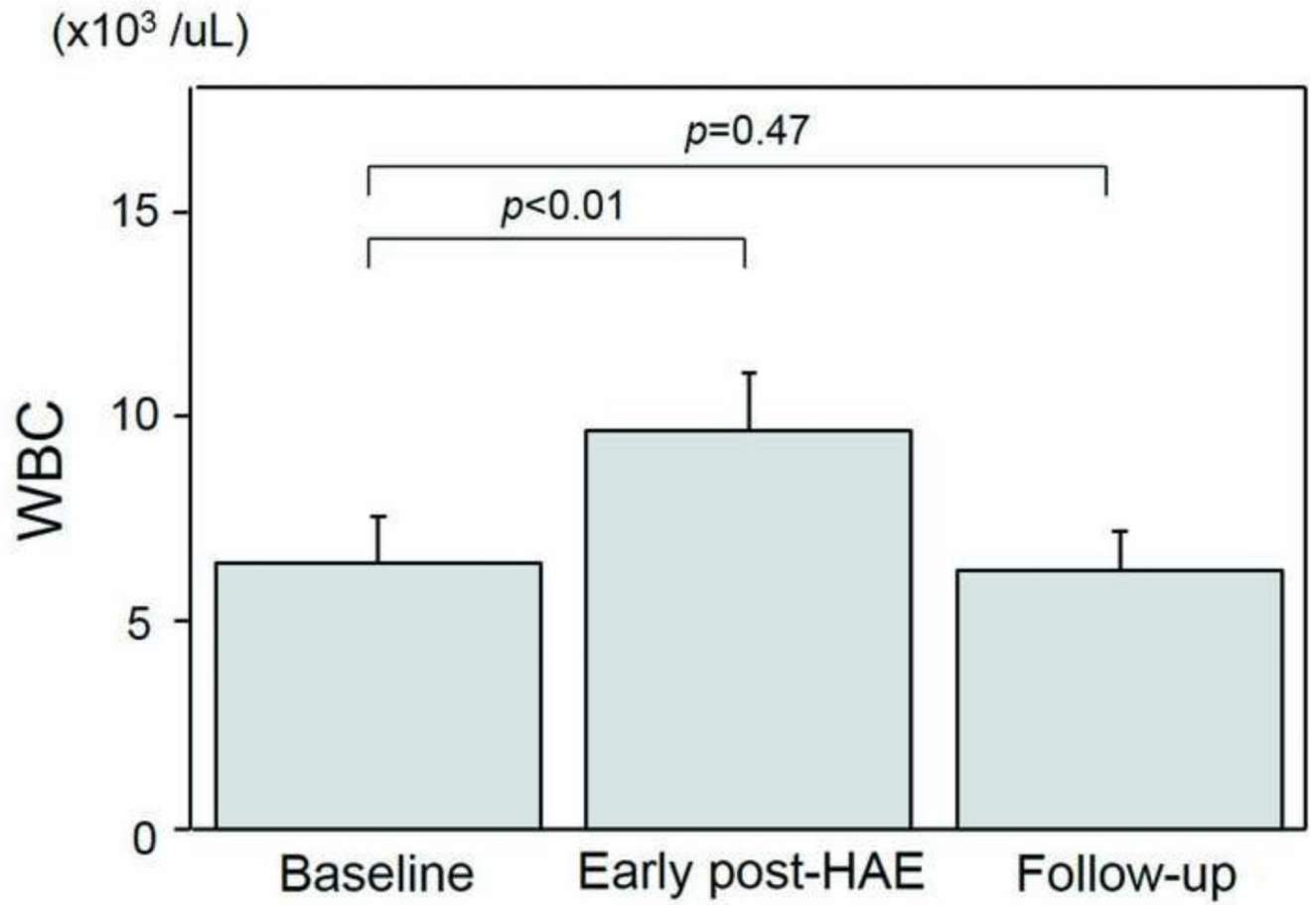
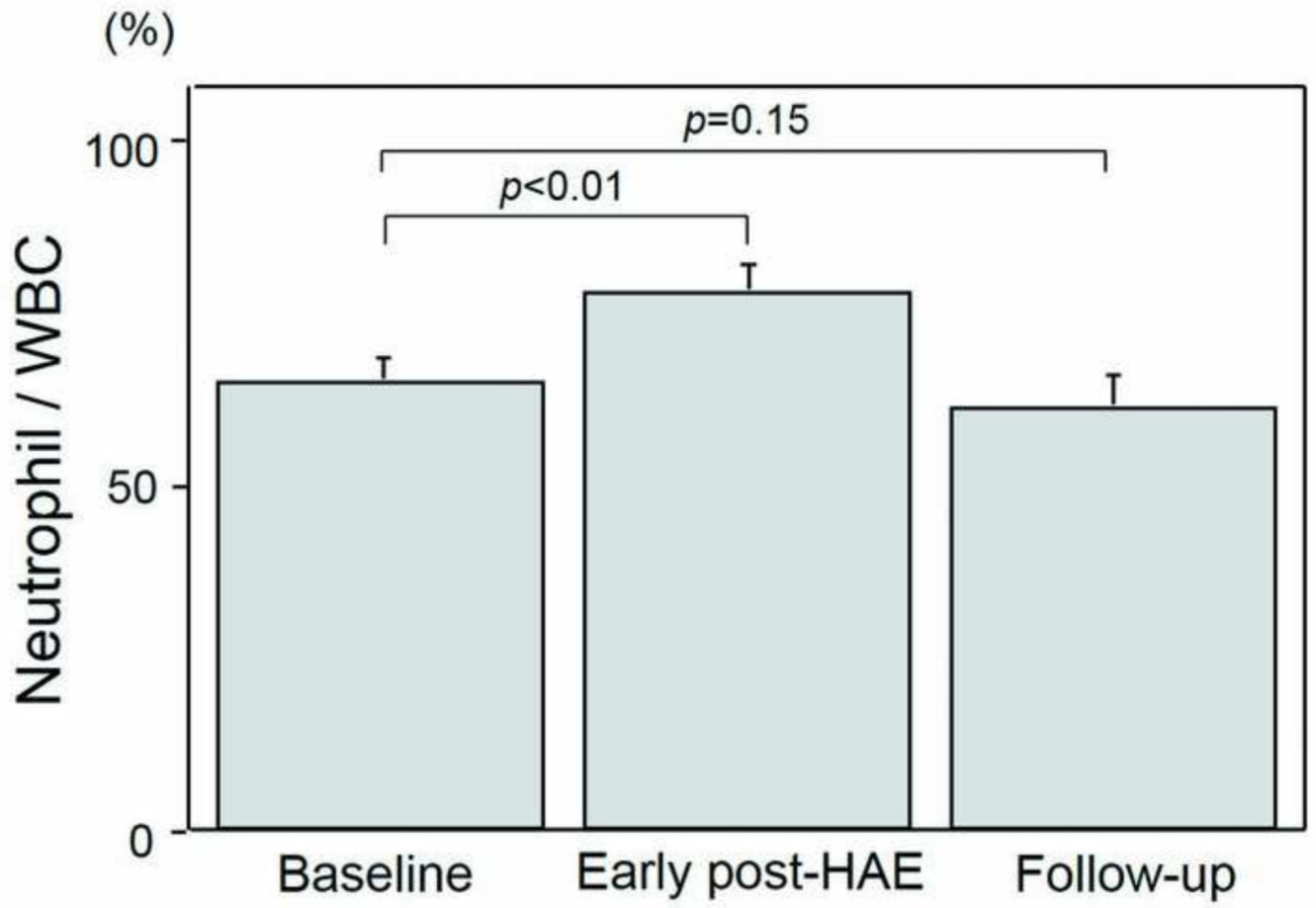


Figure 2. Representative flow cytometry results of a patient who received hepatic artery embolization (HAE) for the treatment of hepatocellular carcinoma. Population of peripheral blood regulatory T-cell (T_{reg}) was decreased (3.5% to 1.4%) in the early post-HAE period. It returned to the baseline level (2.7%) during the follow-up period. CD25, cluster of differentiation 25; FOXP3, forkhead box P3

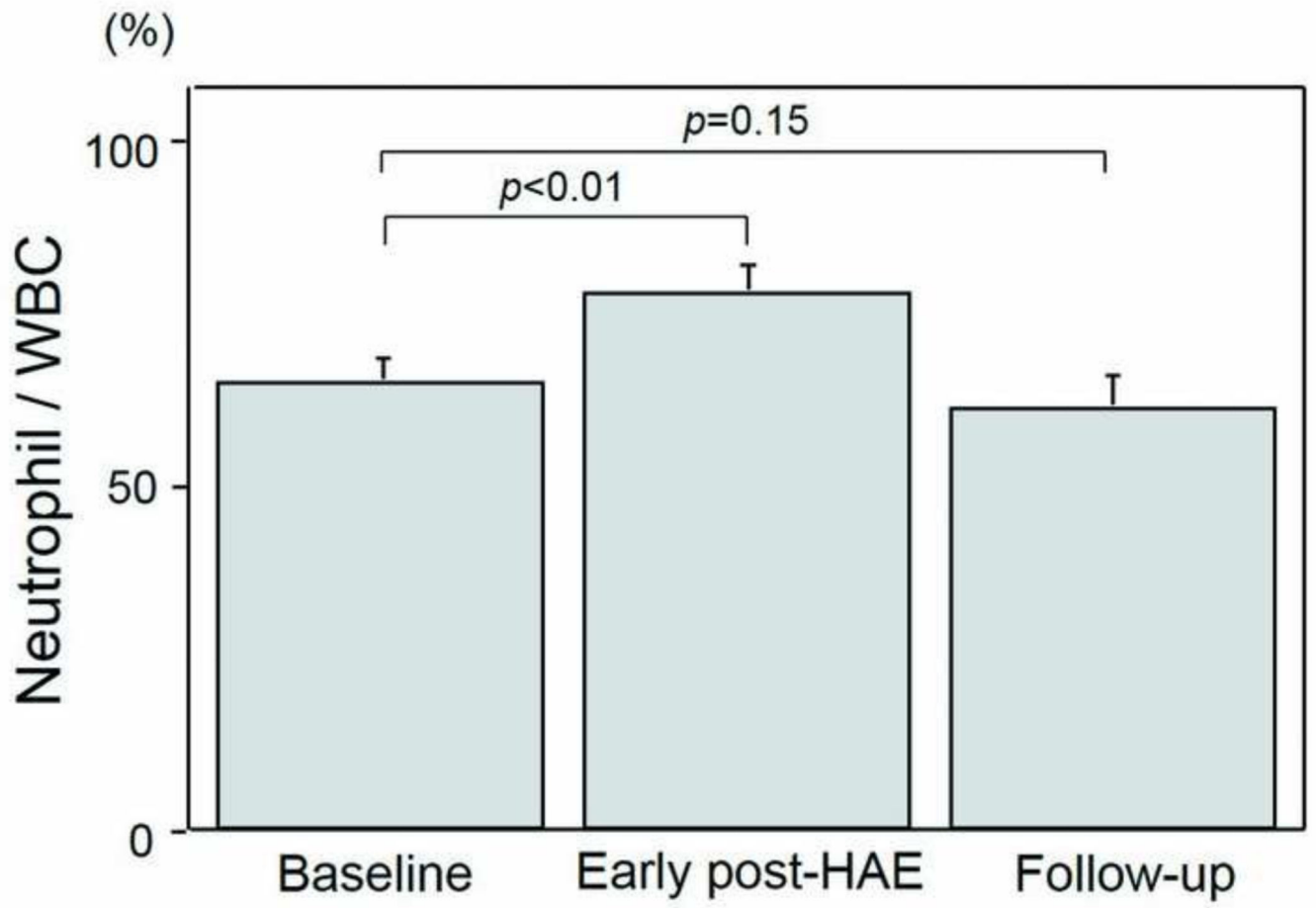
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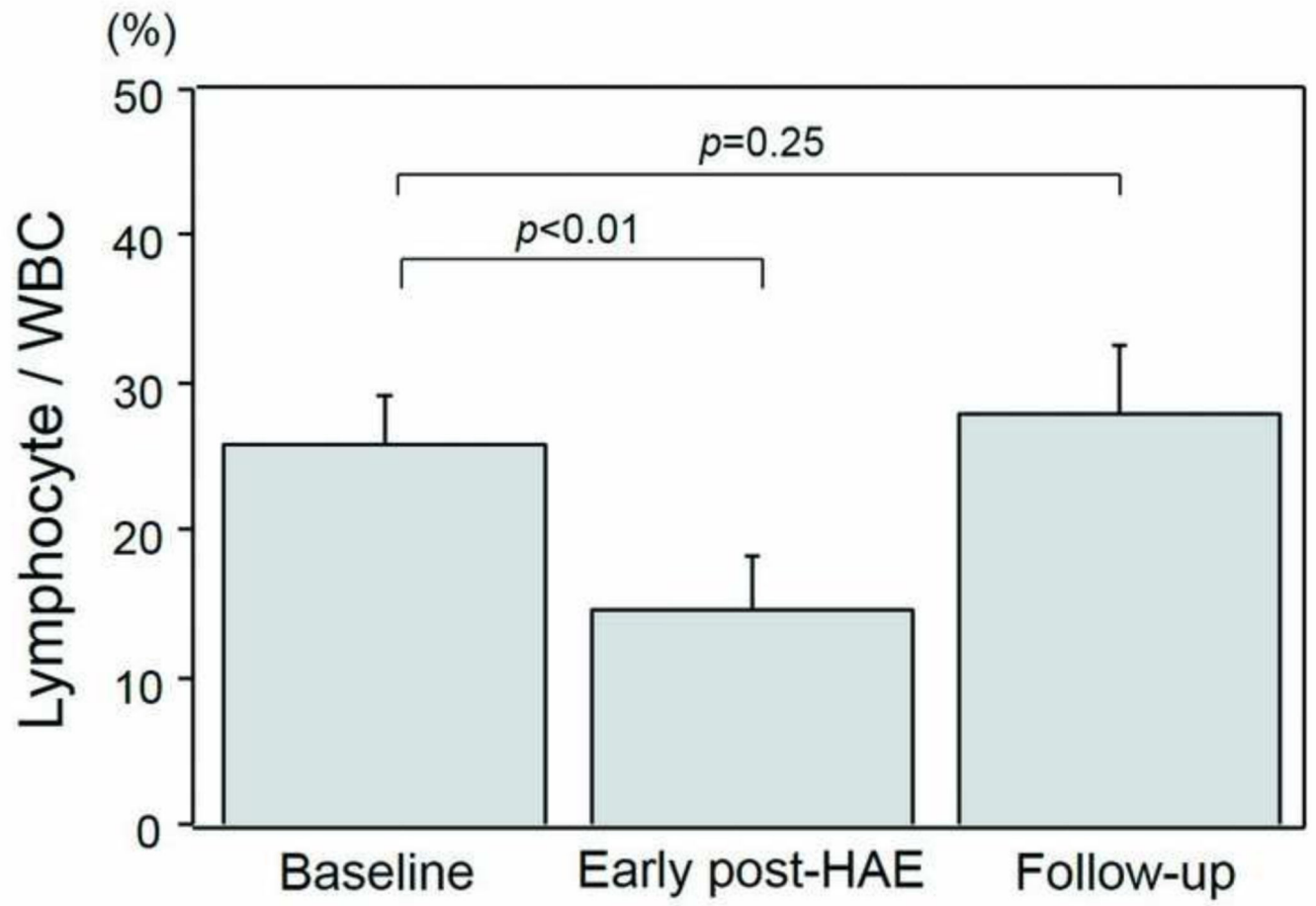
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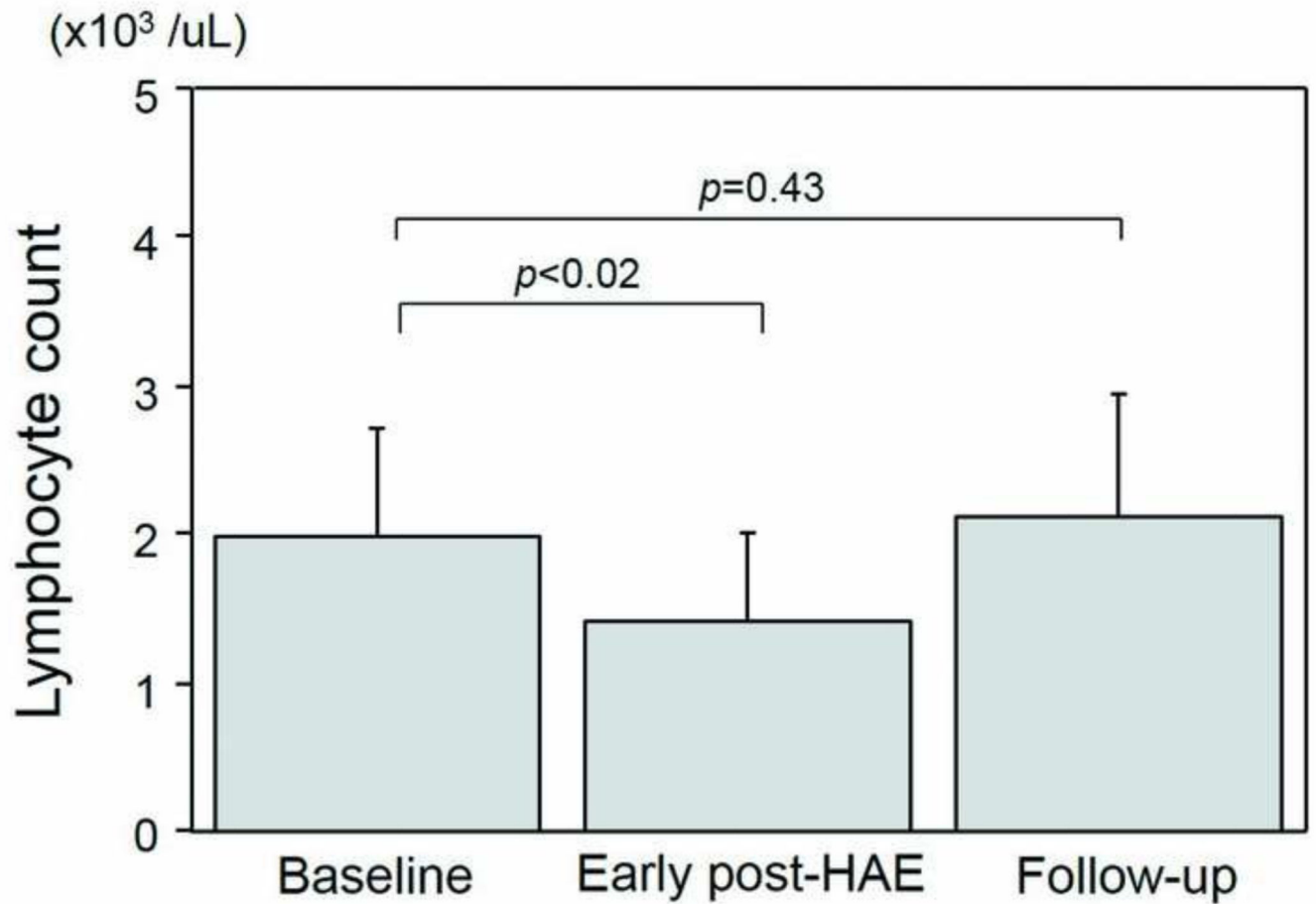
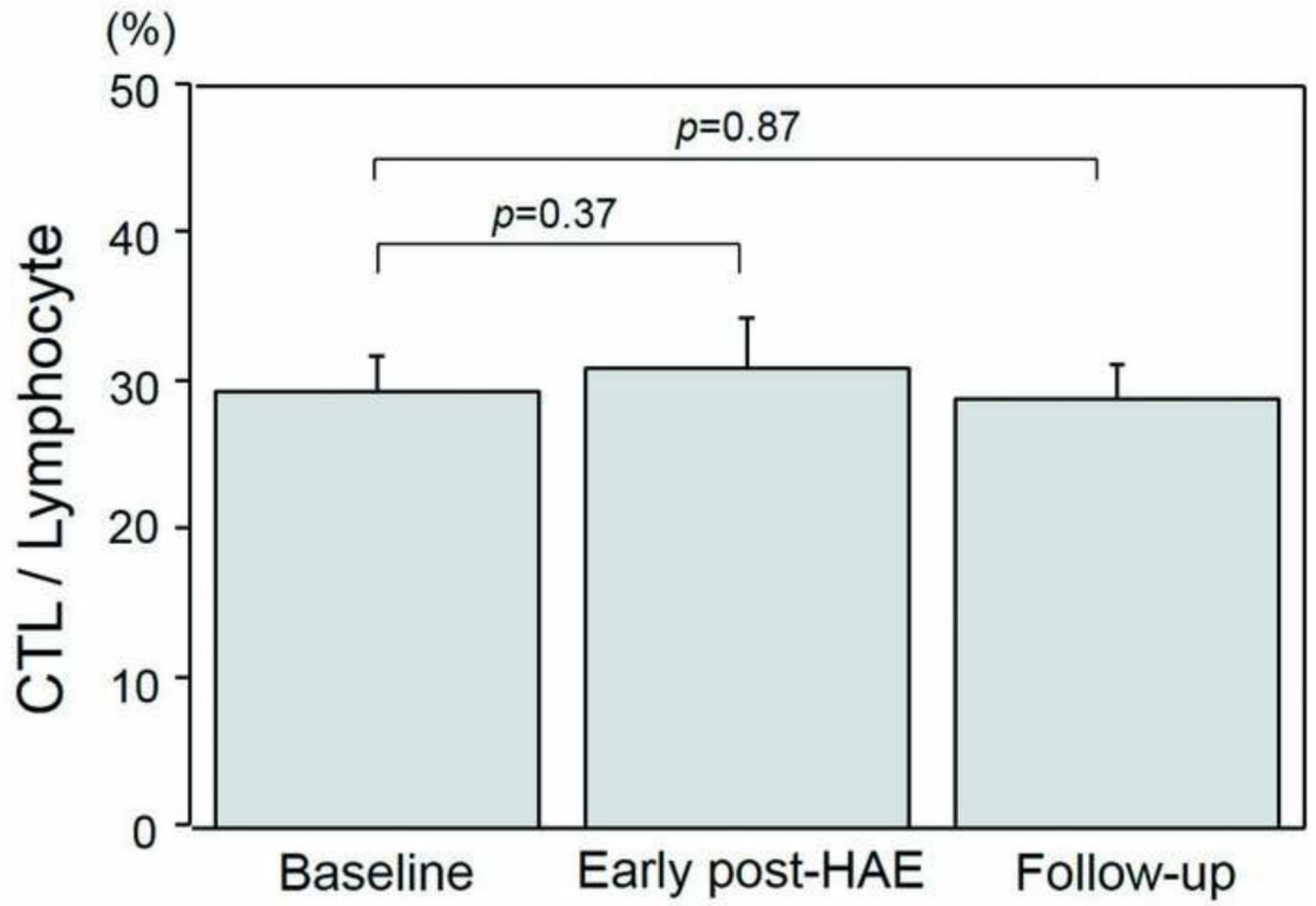
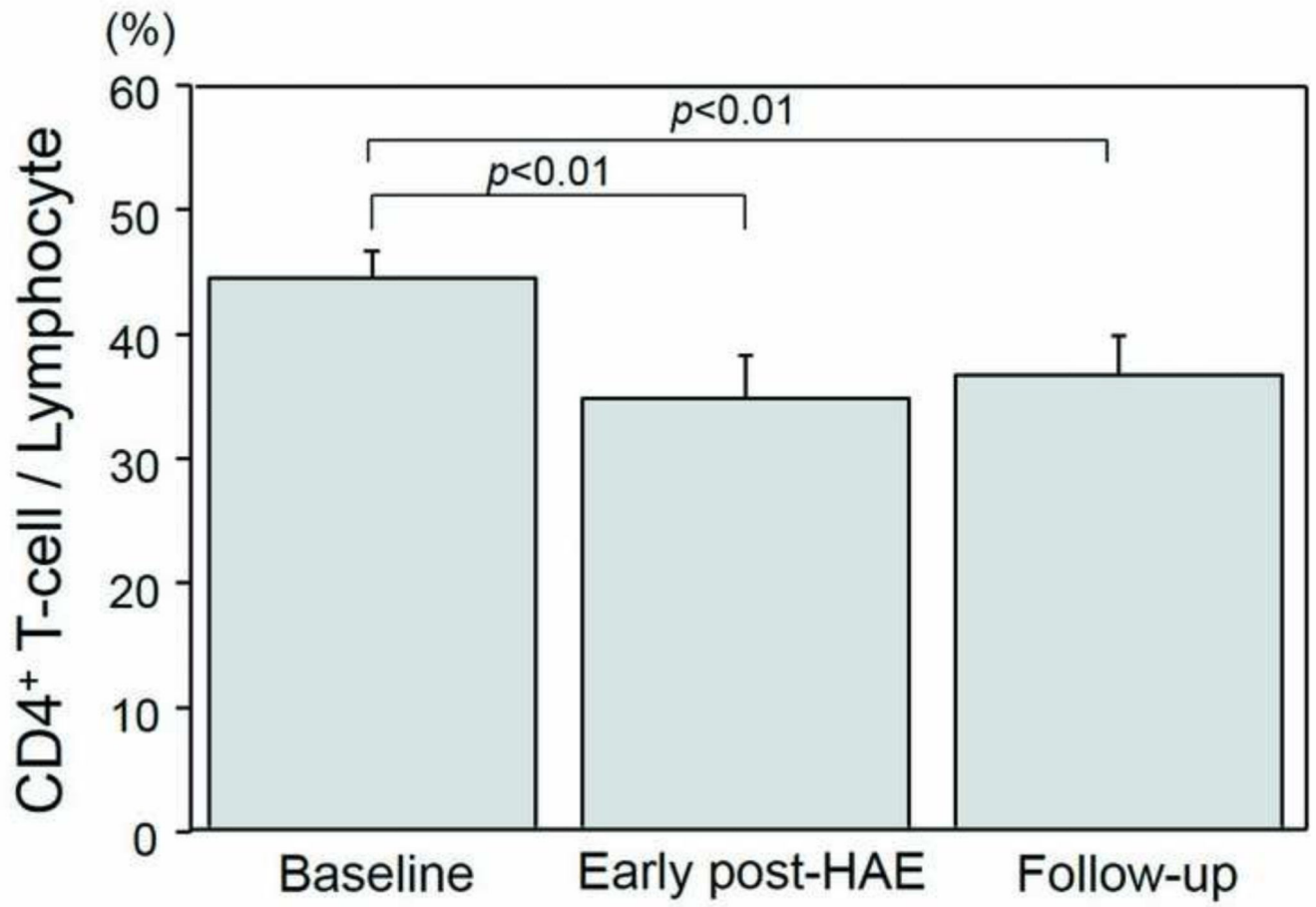


Figure 3. Changes in white blood cells (WBC), neutrophils, and lymphocytes after hepatic artery embolization (HAE). (a) WBC, (b) population of neutrophils in WBC, (c) neutrophil count, (d) population of lymphocyte in WBC, and (e) neutrophil count in peripheral blood sample. Data are expressed as the mean and standard error.

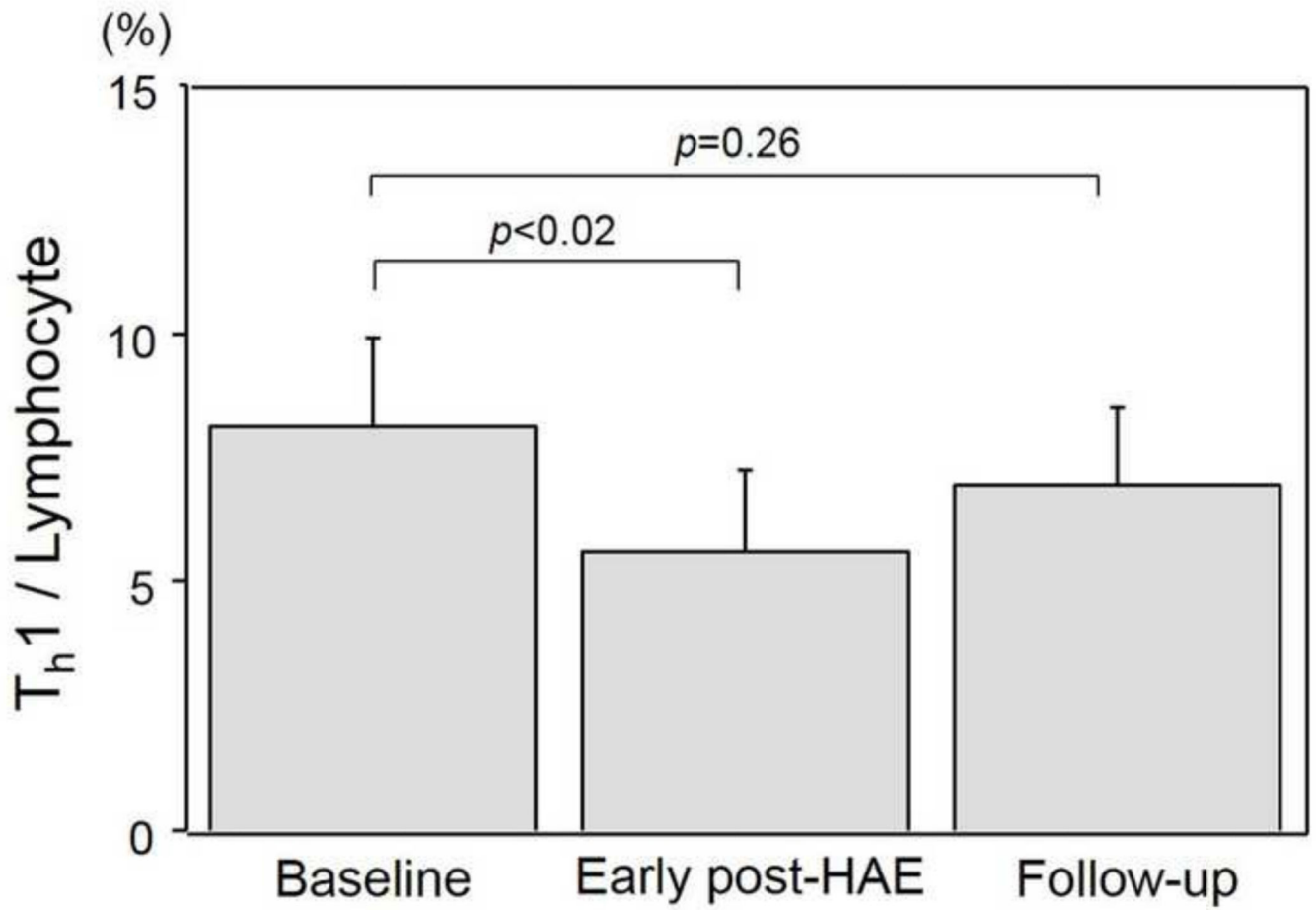
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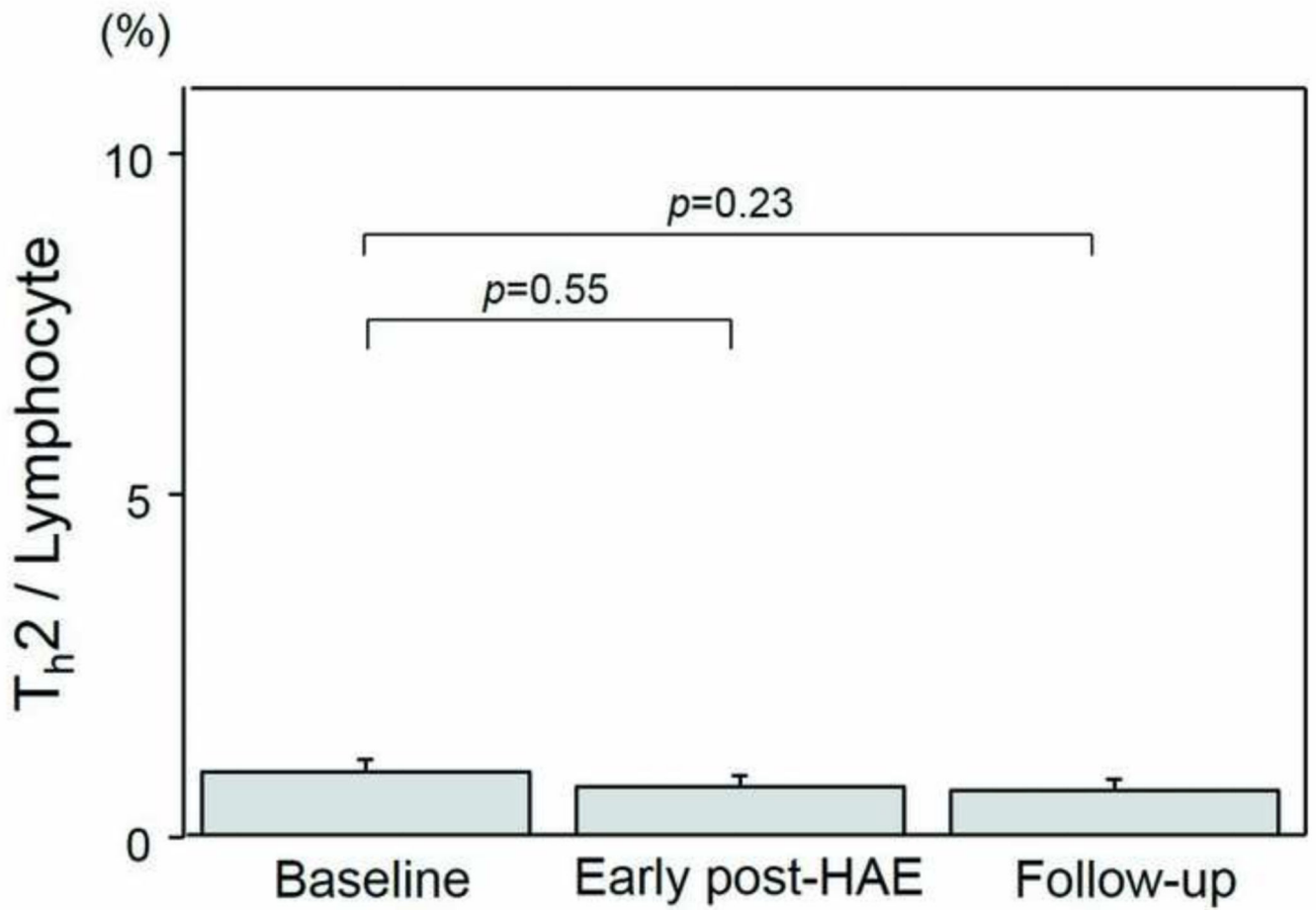
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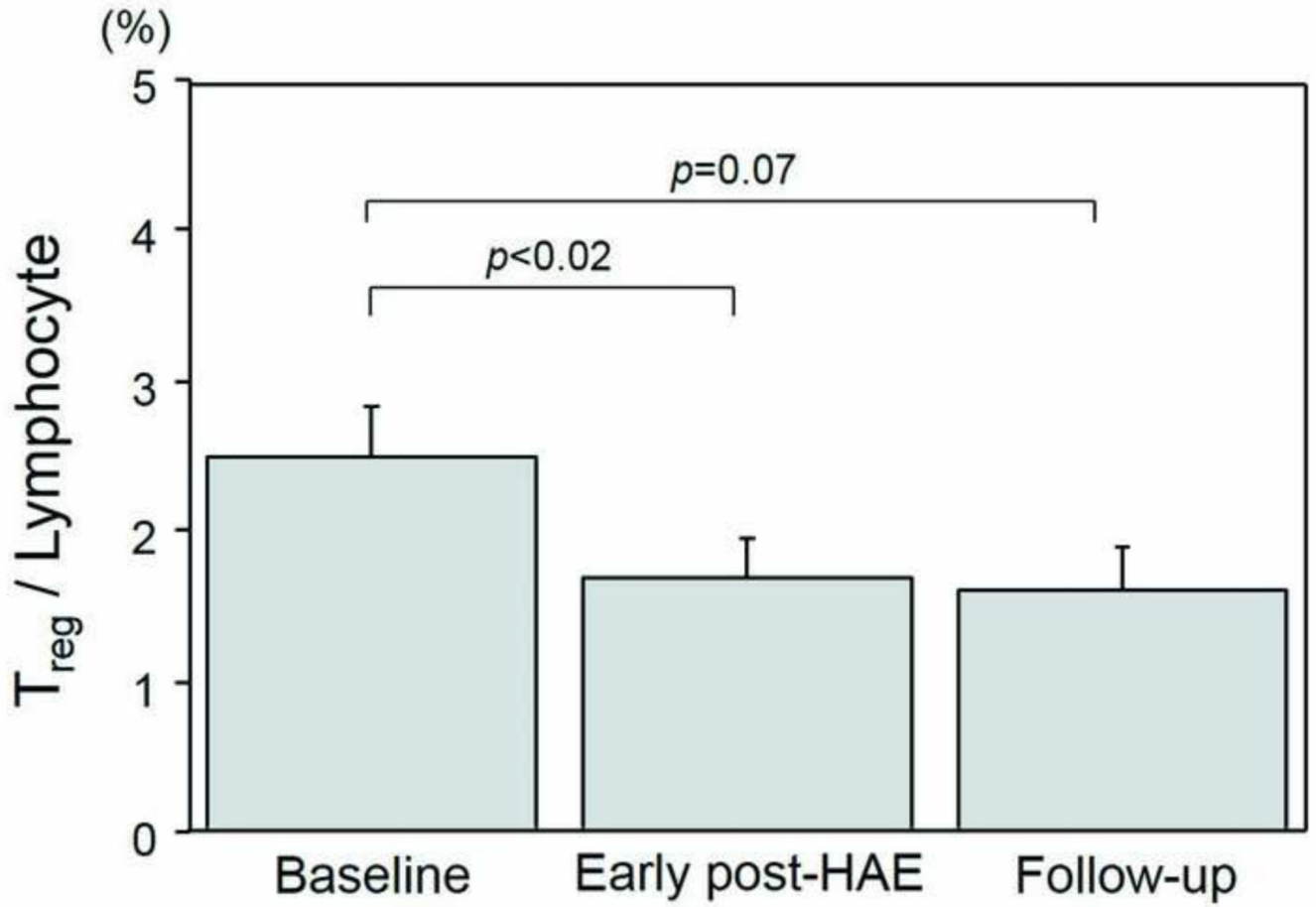


Figure 4. Changes in T-cell population after hepatic artery embolization (HAE). Populations of (a) cytotoxic T-cells (CTL), (b) CD4⁺ T-cells, (c) type-1 helper T-cell (T_h1), (d) type-2 helper T-cells (T_h2), and (e) regulatory T-cells (T_{reg}) in peripheral blood lymphocyte. Data are expressed as the mean and standard error.

Table 1

Patient backgrounds, tumor characteristics, hepatic artery embolization

Parameter	
Patient No.	12
Age (years)	56.2±3.6
60	7 (58.3)
>60	5 (41.7)
Gender	
Male	9 (75.0)
Female	3 (25.0)
Primary diagnosis	
Neuroendocrine tumor	6 (50.0)
Hepatocellular carcinoma	5 (41.7)
Leiomyosarcoma	1 (8.3)
Child-Pugh score	
5	10 (83.3)
6 or more	2 (16.7)
Tumor number	
Single	2 (16.7)
Multiple	10 (83.3)
Maximum tumor diameter (cm)	
5	9 (75.0)
>5	3 (25.0)
Extrahepatic lesion	
No	6 (50.0)
Yes	6 (50.0)
Range of HAE	
Lobar	8 (66.7)
Segmental	4 (33.3)
Maximum AST after HAE (IU/l)	
100	6 (50.0)
>100	6 (50.0)
Initial therapeutic response on mRECIST	
CR or PR	9 (75.0)
SD or PD	3 (25.0)

Continuous data are presented as mean and standard error (SE). Numbers in parentheses are percentages. HAE, hepatic artery embolization; AST, aspartate aminotransferase; mRECIST, modified response evaluation criteria in solid tumours; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

Table 2

Factors affecting fold change of T_{reg} and T_{H1} population

Parameter	Early post-HAE period			Follow-up period		
	Fold change value (T _{reg})	p value	Fold change value (T _{H1})	Fold change value (T _{reg})	p value	Fold change value (T _{H1})
Age (years)		.46			.68	
60	0.65±0.15		0.70±0.12	0.74±0.25		1.00±0.22
>60	0.83±0.15		0.77±0.10	0.88±0.34		0.85±0.10
Gender		.12			.23	
Male	0.81±0.13		0.72±0.10	0.93±0.72		0.95±0.19
Female	0.47±0.08		0.77±0.13	0.39±0.19		0.91±0.18
Primary diagnosis		.22			.57	
HCC	0.89±0.17		0.88±0.12	0.75±0.34		0.93±0.22
Other	0.60±0.12		0.63±0.09	0.83±0.25		0.94±0.18
Child-Pugh score		> .99			> .99	
5	0.71±0.11		0.73±0.10	0.73±0.18		0.93±0.16
6 or more	0.79±0.42		0.73±0.18	1.12±0.95		0.95±0.03
Tumor number		> .99			> .99	
Single	0.79±0.42		0.73±0.18	1.12±0.95		0.98±0.03
Multiple	0.72±0.11		0.73±0.08	0.80±0.19		0.94±0.13
Maximum tumor diameter (cm)		.52			.52	
5	0.76±0.13		0.70±0.09	0.90±0.24		0.89±0.14
>5	0.62±0.19		0.85±0.21	0.48±0.23		1.08±0.37

Parameter	Early post-HAE period			Follow-up period		
	Fold change value (T _{reg})	p value	Fold change value (T _{h-1})	Fold change value (T _{reg})	p value	Fold change value (T _{h-1})
Extrahepatic lesion		< .04 [*]			.34	
No	0.95±0.13		0.86±0.08	0.90±0.26		0.98±0.17
Yes	0.49±0.10		0.53±0.09	0.69±0.31		0.90±0.22
Range of HAE		.61			.23	
Lobar	0.74±0.13		0.76±0.13	0.55±0.11		0.80±0.16
Segmental	0.69±0.20		0.69±0.08	1.28±0.49		1.20±0.17
Maximum AST after HAE (IU/l)		.42			.15	
100	0.62±0.35		0.63±0.09	0.96±0.25		1.08±0.18
>100	0.82±0.16		0.88±0.12	0.63±0.31		0.84±0.20
Initial therapeutic response on mRECIST		.64			.64	
CR or PR	0.70±0.14		0.70±0.09	0.73±0.20		0.81±0.11
SD or PD	0.80±0.10		0.87±0.19	1.01±0.58		1.31±0.38

Data are presented as median and standard error (SE), HAE, hepatic artery embolization; HCC, hepatocellular carcinoma; mRECIST, modified response evaluation criteria in solid tumors; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

^{*} p < .05 as compared with baseline